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Molecular Basis of Thyroid Cancer*

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Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of Nature by careful investigation of rarer forms of disease. For it has been found in almost all things, that what they contain of useful or applicable nature is hardly perceived unless we are deprived of them, or they become deranged in some way.

William Harvey (1657) (1)

I. Introduction

THYROID follicular cell tumors present a unique model for the study of genetic and environmental factors predisposing to benign nodule formation, well differentiated malignant tumors, and anaplastic cancer. Although these histological changes are not necessarily sequential, there is evidence that gradation of proliferative and differentiative potential exists among cells in each thyroid follicle. Conditions conducive to rapid growth ensure that the progeny of cells with high growth potential establish local dominance, a prelude to nodule formation (2). There is also evidence that well differentiated carcinoma may progress to an anaplastic form (3). It is only speculation that benign nodules may develop into well differentiated carcinoma.

Cancer is a complex, multistep process (4). Based on the measurements of age-dependent tumor incidence, it was inferred mathematically that a succession of five or six independent steps are involved, each of which is rate limiting (5). This predicted sequence of events has been shown to apply to colorectal carcinoma (6–12). In the intact host, each step would represent a physiological barrier to be breached for a cell to progress to malignant transformation. The fact that multiple barriers must be overcome ensures that malignancy is a rare event (13).

A consideration of thyroid carcinogenesis raises several issues: What are the cellular events for the transformation of normal thyroid epithelial cells to malignant ones? Are the events that direct transformation of normal thyroid cells to follicular carcinoma and papillary carcinoma separate and independent? How can one account for tumors of mixed histology? How does iodide deficiency enhance the prevalence of follicular carcinoma? Are additional cellular events necessary for the metastatic potential of tumors or is this inherent in those changes resulting in follicular or papillary histology? Similarly, are additional factors involved in the progression of well differentiated carcinoma to anaplastic histology? Is there evidence that benign nodular proliferation is an antecedent to malignant transformation of thyroid cells as has been observed in colorectal cancer?

In this review, an attempt will be made to summarize what is known at the molecular level about thyroid cell growth and the factors involved in the transformation of thyroid cells. The picture is incomplete since what is known about thyroid tumors is not as advanced as for other tumor systems.

II. Thyroid Cell Growth

The thyroid follicular cell is a highly differentiated cell having evolved a highly efficient system for concentrating iodide for the synthesis, storage, and release of a reservoir of thyroid hormones, under basal conditions as well as condi-

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tions of increased need. Thyroid cells are programmed (14) to aggregate into follicles (15) with a central space for the storage of thyroglobulin. This organization necessitates the polarization of the thyroid cell: the basal pole specializes in the import of iodide, which is then transported to the apex for iodination of thyroglobulin (16).

Unlike many other highly specialized cells, thyroid cells are not irreversibly terminally differentiated. When they proliferate in response to certain growth signals they temporarily lose the ability to concentrate iodide and to synthesize thyroglobulin (see Ref. 17 for review). Certain unregulated growth signals associated with malignant transformation may contribute to the loss of differentiated thyroid function. The thyroid cell turns over, on average, no more than five times through adulthood (18). Turnover increases in early infancy and adolescence (18, 19). Because of the inhomogeneity of the growth potential of follicular cells (2), a fraction of these follicular cells in adults would likely turnover much more than five times. It is assumed that cycles of cell division may be more frequent in benign or malignant tumors (2) although evidence from human tumors does not always support this notion (20).

An understanding of the factors involved in normal human thyroid cell growth, differentiation, and signaling is an essential prelude to understanding aberrations related to thyroid cell transformation. Unfortunately, the picture is incomplete due to the variability among species of factors that sustain thyroid proliferation, the pathways used, and cytokines elaborated. Data from animal thyroid tissue in primary culture or from thyroid rat cell lines may not be generalizable to the human thyroid (21–23) (see Ref. 16 for review).

TSH induces human thyroid cell growth at a higher concentration than is necessary for induction of differentiated function (24, 25). TSH mediates its growth stimulation through the adenyl cyclase/cAMP pathway. Insulin or insulin-like growth factor I (IGF-I) synergizes with TSH to induce thyroid cell growth while maintaining specialized cell function (25, 26). Insulin and IGF-I are thus either permissive for other factors without being mitogenic themselves or do not inhibit differentiated function (25, 26). Indeed, TSH has been shown to enhance insulin-induced autophosphorylation of both insulin and IGF-I receptors in rat thyroid cell lines as well as the phosphorylation of the immediate insulin receptor 185 kilodalton (kDa) endogenous substrate (27, 28).

Epidermal growth factor (EGF), on the other hand, induces human thyroid cell growth at the expense of loss in differentiated function (29–31). In some species, TSH induces EGF receptors on thyroid cells, making these cells more responsive to EGF (32, 33). Many EGF effects are reproduced by phorbol esters, used as probes for protein kinase C and diacylglycerol (DAG) (34–36). The effects of TSH, EGF, and phorbol esters on differentiation are largely independent of their mitogenic effects (17). Fibroblast growth factor (FGF) is also a potent thyroid cell mitogen (37).

Growth factors elaborated by thyrocytes have been studied in different species at different times. Human and sheep thyroids produce IGF-I (35, 38), FRTL5 cells produce IGF-II (39), and porcine cells produce FGFs (40); both IGF-I and FGF act upon thyrocytes in an autocrine fashion.

Likewise, cytokines traditionally thought to be synthesized and secreted by immunocytes are synthesized, secreted, and act upon thyrocytes. These cytokines include interleukin-1 (IL-1), transforming growth factor β (TGFβ), and IL-8. They either have growth-promoting effects as in physiological concentrations of IL-1 and IL-8 (41–43) or inhibit growth and in some instances differentiated function, *e.g.* TGFβ, interferon-γ, and IL-1 (44–47). Indeed, TGFβ may have a physiological role in negatively regulating thyroid follicular cell function. IL-1 has been shown paradoxically to limit the growth of human thyroid tumor cell lines (48). Several of these cytokines elaborated by thyrocytes synergize with each other and with growth factors (42, 45) in their effect on thyroid cell growth and differentiated function.

Many of the factors elaborated by the thyroid, *e.g.* IGF-I (38) and FGF (40) also act *in vivo* on endothelial cells and fibroblasts to induce neurovascularization (49) and to enhance adhesion molecule synthesis to support thyroid growth.

At least three distinct pathways for signal transduction have been defined in the thyroid: 1) receptor/adenylate cyclase/protein kinase A system; 2) receptor/tyrosine kinase pathway; and 3) receptor/phospholipase C cascade (50, 51). TSH activates both the adenylate cyclase and phospholipase C pathways. Activation of phospholipase C results in the formation of DAG and inositol-1,4,5-triphosphate (IP_3) (52, 53). DAG activates protein kinase C and IP_3 increases intracellular calcium concentrations. The concentration of TSH necessary to activate phospholipase C is much higher and its action slower than that necessary for adenylate cyclase activation. Apparently cAMP induces DAG synthetase in the thyroid, thus providing substrate for protein kinase C, allowing the activation of this pathway at physiological TSH concentrations (54).

EGF, insulin, and IGF-I act through tyrosine protein kinase receptors (17). EGF also mobilizes calcium (Ca^{++}) from intracellular stores as well as from extracellular sources through the generation of IP_3, at least in some species (17, 55, 56), and perhaps through other mechanisms.

A role for iodide in the regulation of thyroid cell growth has been proposed. Apparently through oxidized intermediaries, iodide may decrease both adenylate cyclase and Ca^{++} levels in thyroid cells, thus reducing their sensitivity to TSH signaling (57–61). Iodide deficiency, conversely, enhances the effects of TSH on the thyroid.

TSH induces or increases phosphorylation of at least 11 proteins. EGF induces the phosphorylation of five proteins including mitogen-activated protein kinase, which in turn phosphorylates ribosomal S6 kinase ($pp90^{rsk}$) (62–64). Phorbol esters induce the phosphorylation of 19 proteins. No overlap exists between the proteins phosphorylated through the two pathways (TSH and EGF/phorbol esters) (62).

The divergence of the two pathways, TSH/cAMP and that of the EGF/kinase C, also extends to newly synthesized proteins. Only one of the 26 proteins involved, proliferating cell nuclear antigen, is synthesized in response to TSH, EGF,

and phorbol esters. There are, however, differences in the kinetics of proliferating cell nuclear antigen synthesis—early through the TSH/cAMP pathway, and delayed through the other two pathways (63).

TSH induces rapid but short-lived increases in c-*myc* transcripts, whereas c-*fos* transcription is slightly delayed. EGF induces c-*myc* gene transcription later than does TSH. The kinetics of c-*fos* messenger RNA (mRNA) synthesis are, however, similar for TSH, EGF, and phorbol esters (65, 66). After an initial increase in c-*myc* mRNA in response to TSH, these transcripts decrease below control values, apparently related to a negative transcriptional signal (67). c-*myc* Gene activation is thus tightly regulated in its role in thyrocyte growth.

III. Epidemiological and Clinical Considerations

Thyroid carcinoma is an infrequent tumor with geographic variation in its prevalence. The annual rates of newly diagnosed cases vary from 0.9/100,000 men and 2.4/100,000 women in Britain (68) to 2.1/100,000 men and 5.2/100,000 women in the United States (69). Thyroid cancer accounts for 0.6% and 1.6% of all cancers among men and women, respectively, in the United States (70). In Japan the combined estimate is 1.4/100,000 of the population (71), whereas the crude relative frequency of thyroid cancer among Kuwaiti females was reported as 10.5% (72).

The incidence of thyroid cancer in the United States increased during the four decades 1935–1975, reflecting better diagnosis as well as the emergence of radiation-related cancers (73–75). That trend has stopped (76). Moreover, since the 1960s, survival rates for white patients have steadily increased (77).

Papillary carcinoma accounts for 85% of differentiated thyroid follicular cell cancers in iodide-sufficient countries (78), while the remaining epithelial thyroid cell tumors are predominantly follicular carcinomas. Anaplastic carcinoma, whose incidence has been decreasing, often arises from preexisting well differentiated (usually follicular) carcinoma (79, 80). An increase in the prevalence of follicular carcinoma under conditions of iodide deficiency or endemic goiter accounts for the overall increased incidence of thyroid cancer, including anaplastic carcinoma, in such areas (81–84a). Incidental microscopic (occult) to small papillary carcinomas are detected with such high frequency at autopsy (85) that one must conclude that the vast majority are not clinically relevant. The factors determining the transition from microscopic foci to detectable thyroid carcinoma are unknown.

Both cohort and case-control studies have established a strong link between external radiation and benign as well as malignant thyroid tumors (78, 86–99). By contrast, internal radiation probably does not enhance the risk of thyroid cancer (88, 100–102).

The relationship of thyroid cancer to endemic goiter has long been suspected (103). Comparative studies of thyroid cancer incidence between areas with and without endemic goiter have not, however, always supported that notion (104, 105). The effect of iodide supplementation on thyroid cancer incidence has also yielded conflicting results (106). Indeed, a recent case-control study from Italy suggested that the influence of iodide supplementation on cancer incidence was marginal (105). It is unclear whether the association of thyroid cancer with benign nodules and goiter is real or related to ascertainment bias (89, 92, 94). Likewise, the notion that thyroid carcinoma is more frequent in patients with Graves' disease, and tends to be more aggressive (107–109), has been challenged (110, 111).

Several host factors that determine the outcome of thyroid cancer have been identified. These include patient age, tumor size, histological appearance, local invasion, lymph node and distant metastases, and aneuploidy (112–114). Several clinical staging and prognostic systems have been based on these factors.

IV. Genetic Factors

Genetic factors are not generally thought to be important in predisposing to thyroid follicular carcinoma. Thyroid carcinoma is, however, increased in such syndromes as Gardner's syndrome (adenomatosus polyposis) (115–121) and Cowden's disease (hamartomas) (122). In addition, reports of remarkable aggregation of papillary thyroid cancers in families (123–125) indicate that genetic factors may be relevant to thyroid follicular cell cancer. In a genetically homogeneous population, 3.8% of patients with papillary cancer had similarly affected family members (126). Genetic factors appear to be more important in the genesis of papillary carcinoma (123, 124, 126) compared to follicular carcinomas (see Ref. 127 for review; 128). Familial aggregation of follicular carcinoma is documented in families with dyshormonogenesis (129). Female patients with papillary carcinoma (but not their relatives) are at greater risk for other cancers [including breast, renal, and central nervous system malignancies (126, 130–133)].

There are also indications that certain ethnic groups may be more susceptible to the effect of ionizing radiation. Furthermore, in instances where siblings were irradiated, the incidence of thyroid tumor development was more than could be accounted for by chance (134).

The association of thyroid cancer with certain HLA alleles represents an interplay between the environment and genes in its pathogenesis, and has been found only in some populations. While no association of HLA antigens with well differentiated thyroid cancer was found in patients from an iodide-sufficient area (Newfoundland) (135), HLA-DR1 was significantly increased in patients from iodide-deficient Eastern Hungary (136, 137). The increase in HLA-DR1 was greatest in patients with follicular cancer (137). Sridama and colleagues (138) in Chicago reported an association of thyroid cancer and HLA DR7, particularly in patients with follicular carcinoma (138). Moreover, immunoglobulin G heavy chain allotypes interacted with HLA to enhance the risk conferred by the latter (139).

To account for these variations in HLA association with thyroid cancer in different geographic locations, it has been suggested that HLA DR1 positive individuals were at increased risk for thyroid cancer in iodide-deficient areas and that this predisposition disappeared once iodide sufficiency predominated (127, 137). In the absence of iodide deficiency,

other HLA-linked influences were important for those exposed to other environmental hazards. It is interesting that the susceptibility of rats to diethylnitrosamine carcinogenesis is related to the inactivation of recessive genes that map to MHC (140) and that nitrosamines induce thyroid tumors in mice (see also *Section VIII.A.*).

Last, the 3-fold greater incidence of thyroid cancer in females is probably related to the female hormonal milieu rather than to the contributions of active genes on the X chromosome. A case-control study (74) has suggested an association of thyroid cancer with pregnancy,

The cellular genetic events leading to thyroid cancer are somatic in nature and thus restricted to thyroid tumor tissue. The influence of paternal imprinting (141, 142) on oncogene activation is unknown.

V. The Cell Cycle and Cancer

Multicellular organisms have highly coordinated mechanisms to control cellular interactions. These signaling networks mediate normal embryonic development and the response to wound healing or infection. Aberrations in growth factor signaling pathways are intimately linked to cancer (143). Malignant cells arise as a result of a stepwise progression of genetic events that include the unregulated expression of growth factors or components of their signaling pathways.

Individual cells receive two classes of signals. One type promotes cell growth largely through the elaboration of growth factors. Another type of signal allows cells to inhibit the growth of their neighbors through regulatory proteins. The known number of genes involved in control of the cell cycle is increasing rapidly. When the function of some genes are lost, cells become unresponsive to growth-inhibitory signals. Several of these genes meet the requirements for "tumor suppressors." The inhibitory signals may involve not only proteins that function as switches in the cycle, but also diffusable growth inhibitors and hormones (144–146). The cell responds to inhibitory signals in three ways: 1) pausing in a certain phase of exponential growth often representing arrest at the G_1-S phase transition; 2) postmitotic differentiation; 3) senescence or apoptosis (programmed cell death) (147).

Growth signals allow cells in the resting (G_o) phase to enter and proceed through the cell cycle. The resting cell is first advanced into the G_1 phase of the cell cycle by "competence" factors, traverses the G_1-S phase and becomes committed to DNA synthesis under the influence of "progression" factors. Although the notion of "competence" was only demonstrated in some systems, its generalization is probably justified. Transition through the G_o-G_1 phase requires sustained growth factor stimulation over several hours. Disruption of the signal for only a short period of time results in the cell reverting to the G_o phase. There is also a restriction point in the G_1 phase during which the presence of both competence and progression factors are necessary for the cell to advance through its cycle (Fig. 1). Thereafter, only progression factors are needed. Competence factors include EGF and FGF whereas IGF-1 and insulin act as progression factors (143). The cytokines such as TGFβ, interferon-γ, and tumor

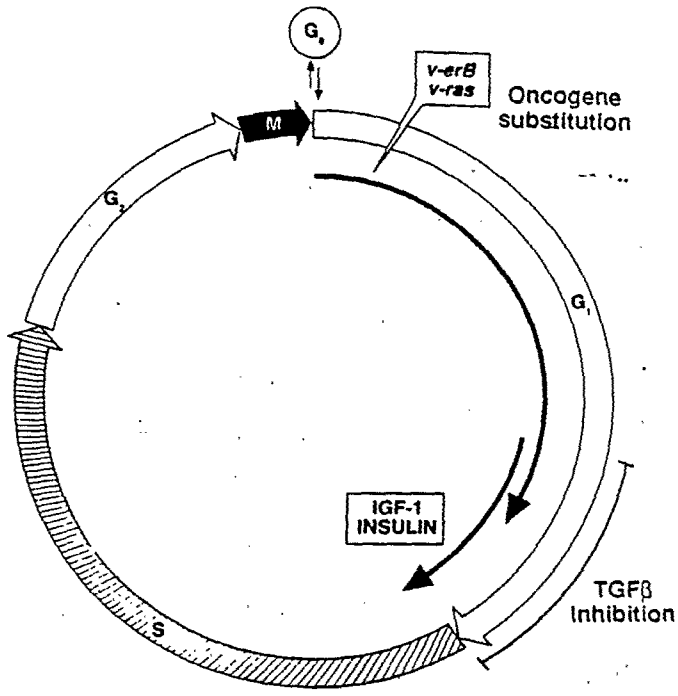

FIG. 1. The cell cycle. Sites of action of protooncogenes and growth and growth-inhibiting factors. Some protooncogenes, *e.g. c-ras* and growth factors such as EGF, contribute to the cell "competence" and are complemented by the action of others, *e.g.* insulin, to allow progression of the cycle beyond the G_1-S restriction phase, thus committing the cell to DNA synthesis. For human thyroid cells TSH is a "competence" factor. Both retinoblastoma (Rb) and p53 proteins act at the G_1-S restriction phase to halt cell cycle progression. One of the pathways of Rb growth-inhibiting influence is through the induction of the TGFβ gene [Adapted with permission from S. A. Aaronson: *Science* 254:1146–1153,1991 (143). ©1991 by the AAAS.]

necrosis factor can antagonize the effects of growth factors. Activated oncogenes may encode growth factors (*e.g. c-sis*), receptor protein kinases, or other enzymes that participate in mitogenic signaling. Oncogenes such as *c-ras* and *c-erbB* function as competence factors. Other oncogenes, *e.g., bcl-2* (148–150), act by blocking apoptosis and allowing cells to proliferate preferentially in response to mitogenic signals and may indeed block the apoptotic affects of *c-myc* (151–153). p53 And retinoblastoma (Rb) proteins function by regulating the cycle at the critical G_1 to S phase transition (154–156). Several of the growth factors participate in tumor progression by providing in a paracrine fashion the environment for proliferation, invasion, and metastases of epithelial cells. A number of growth factors including FGF, EGF, and hepatocyte growth factor participate in tumor-sustaining neoangiogenesis.

Are tumor cells intrinsically more unstable than normal cells, or do they merely acquire their abnormal karyotypes through increased division and intense selection at *normal* rates of chromosomal rearrangement? This critical question has been definitively answered only recently (157). Many tumor cells exhibit rates of gene amplification several orders of magnitude greater than normal cells. Gross chromosomal changes such as marked aneuploidy, translocations, or dele-

tions are not nearly matched by the rates of point mutations (158). Such genomic instability is a genetic trait inherited in human cancer-prone syndromes (159, 160). These may be associated with a defect in G_1-S phase and is upstream from the p53 gene in a pathway that activates the G_1-S checkpoint. X-ray exposure of eukaryotic cells results in G_1 arrest and is associated with an increase in p53 protein. Cells lacking p53 or having a dominantly negative mutation lack this G_1-S delay (161). In premalignant cells, increased genetic instability after DNA damage related to radiation, carcinogens, or single strand breaks results in duplication, amplification, and replication of broken sister chromatids (157, 161). Some of these events may result in the loss of growth suppressor genes and amplification of oncogenes, although the picture is likely more complex (162, 163). This route for carcinogenesis is worthy of study in radiation-related thyroid tumors.

VI. Thyroid Cell Cancer—Mono or Polyclonal?

Recent work involving mutated *ras*, GSα (164), and rearranged RET/PTC (PTC = papillary thyroid carcinoma oncogenes) (165) have indicated variations in the presence of mutant gene products within the same tumor tissue. Whether these abnormalities are secondary or primary in origin, or related to multiple foci each arising with a separate array of mutations, are pertinent questions, and raise the issue of the clonality of thyroid tumors. If tumors arise by a series of rare mutations or epigenetic events, it is implied that such lesions derive from a single cell.

Determination of clonality can be achieved by X chromosome inactivation analysis, taking advantage of the fact that one of two X chromosomes is functionally inactivated early in mammalian embryogenesis (166). X-inactivation is random involving either the paternal or maternal X chromosome so that female tissues are mosaics containing an equal mixture of cells in which maternal or paternal chromosomes have, respectively, been activated. Taking advantage of differences in restriction fragment length polymorphisms (RFLPs) between two alleles in heterozygote females and the ability of some restriction enzymes to identify the methylation differences (167) between the functional and inactivated genes, the clonality of benign and malignant thyroid tumors has been studied (168–171). Using RFLPs at the hypoxanthine phosphoribosyltransferase, phosphoglycerokinase (PGK), and more recently M27 marker genes (171), most benign nodules and malignant thyroid tumors were shown to be monoclonal in origin. Nine of 13 rapidly growing nodules within 12 multinodular goiters were also found to be monoclonal using the highly informative M27 marker (171). One hyperfunctioning thyroid nodule in a 22-month-old child was polyclonal (172).

A problem in this analysis of solid tumors is the potential contamination of tumor samples by nontumorous tissue. The polymerase chain reaction (PCR) may facilitate the analysis for monoclonality of small homogenous tissue fragments (173): *Hpa*II digestion of DNA followed by PCR amplification with primers on each side of *Hpa*II restriction site within the PGK gene would allow the amplification of only the methylated copy of the gene (174). Random inactivation of PGK is associated with two DNA fragments whereas monoclonality is associated with one band.

VII. Growth Factors and Their Receptors in Thyroid Tumors

Unregulated growth signals acting on cells with multiple transforming lesions result in malignancy. These signals may be the result of constitutive synthesis of growth factors, constitutive activation of their receptors, or of switches in the signal transduction pathways. It is thus of interest to review what is known in this context of thyroid carcinoma (175).

EGF and EGF receptor have been detected by immunohistochemistry in malignant thyroid tumors, but not in normal thyroid tissue, benign tumors, or multinodular goiter (176, 177). The presence of both the growth factor and receptor was more frequent in papillary than in follicular carcinoma samples (176). EGF receptor was also detected by radioligand assays in normal and hyperplastic thyroid tissue, as well as benign and malignant tumors (178). In that study, the level of EGF receptor was found to be greatest in anaplastic tumor tissue (178). The predominance of EGF receptor expression in papillary carcinoma was extended to the transcripts of c-*erbB1* and c-*erbB2/neu* oncogenes, which encode EGF receptor or analog, respectively (179, 180). A 2- to 3-fold increase in c-*erbB1* and c-*erbB2/neu* mRNA in papillary carcinomas and their lymph node metastases, as well as in one benign adenoma, compared to thyroid tissue were reported (180). No structural abnormalities in these protooncogenes were detected in a large number of benign and malignant thyroid tumors (179, 181).

EGF expression was shown to be of prognostic value in that tumors with higher EGF expression were more likely to recur (177), analogous to the findings with c-*erB1* and c-*erbB2/neu* (180).

No evidence for rearrangement or mutation of c-*erb*A (T_3 receptor) was found in thyroid tumors (179). At least some mutated T_3 receptors interfere with T_3 action (as well as perhaps that of related receptors in a dominant manner (Refs. 182–184 for review), which makes them potential candidates for tumor suppressors (147).

Analysis by immunohistochemistry revealed TGFβ expression in 58% of malignant thyroid tumors, but not in benign adenomas or normal thyroid epithelium (185). In follicular carcinomas TGFβ immunostaining was related to the presence of H-*ras* 61 (Glu → Arg) mutation. The fact that TGFβ is a potent inhibitor of epithelial thyroid cell growth and is synthesized by thyroid cells suggests that the increase in TGFβ may compensate for decreased TGFβ sensitivity (186, 45). TGFα appears to regulate the expression of EGF receptor in some tumors and thus promotes their growth (187), but was not studied in thyroid cancers. Apparently both benign and malignant thyroid tumors elaborate IGF-I (38, 188) more than normal tissue, which may allow nodules to become autonomous (26).

In anaplastic thyroid carcinoma, receptors not normally expressed in the normal thyroid gland may be detected. Functional but aberrantly glycosylated receptors for platelet

derived growth factor (PDGF) were described in an anaplastic thyroid carcioma cell line (189).

VIII. Oncogenes Involved in Thyroid Carcinogenesis

A. *ras*

The mechanisms by which receptor tyrosine kinases stimulate specific intracellular signaling pathways have been elucidated by the identification of a conserved protein motif of the *src* homology 2 (SH2) domain, which is found in a remarkably diverse group of signaling proteins (190). This includes nonreceptor protein kinases, phospholipase C-γ1, *ras* guanine triphosphatase (GTPase) activating protein (GAP), and other molecules referred to as adaptors. Proteins with SH2 domain frequently have another distinct sequence, the SH3 domain, which is also implicated in the regulation of protein-protein interaction during signal transduction. For example, the autophosphorylation of the insulin receptor results in tyrosine phosphorylation at the SH2 consensus sequence of a 185 kDa cytosolic protein (27), which then recruits and activates SH2-containing phosphatidylinositol (PI) 3′-kinase (191). A similar scheme may be envisaged for GAP, which upon phosphorylation associates through SH3 domains with guanine nucleotide exchange factors (190, 192), thus regulating *ras* and *ras*-like G proteins. Moreover, tyrosine protein kinases, through SH2-containing protein adaptors (193, 194) may activate *ras*-dependent signaling pathways (Fig. 2). The intermediate elements in this cascade have been recently elucidated, at least in some settings (see Ref. 195 for review).

GAP stimulates the GTPase activity of *ras* which is a critical component of intracellular mitogenic signaling pathways (194, 196) and also acts as a negative regulator of *ras* function (197). Mutations that cause oncogenic activation of *ras* lead to accumulation of *ras* bound to GTP, the active form of the molecule (196). These mutations block the ability of GAP to promote conversion of *ras* to its inactive, GDP-bound form (194). GAP may also function in a complex with *ras* as an effector of its downstream signaling functions (198). Thus, mutations that impair interaction of *ras* with GAP also block the biological function of *ras*. *ras* Protein (p21) is probably excited by an activator upstream in its signaling pathway and passes these signals to a downstream effector pathway (Fig. 3). It is conceivable that constitutive activation or amplification of the signals acting along *ras* or *ras*-like pathways may be involved in cell transformation. It is also possible that mutations in the SH2 adaptors may have similar consequences. The signals involved in the increased expression of *ras* during normal thyroid cell growth (199) are unknown.

ras Protooncogene mutations are found in more than 30% of human tumors (200). Three families of *ras* proteins have been identified, Ha-*ras*1, K-*ras*2, and N-*ras*, each of which is located in a separate chromosomal region (196). All three are subject to mutations, although some mutations tend to predominate in specific tumors, *e.g.* K-*ras* mutation at codon 12 in pancreatic carcinoma (201) and adenocarcinoma of the lung (202). Mutations at residues 12, 13, or 61 of any of the *ras* protooncogenes convert them into active oncogenes (196); other mutations may also be relevant to constitutive signaling. Mutations at codon 61 are the most efficient in changing

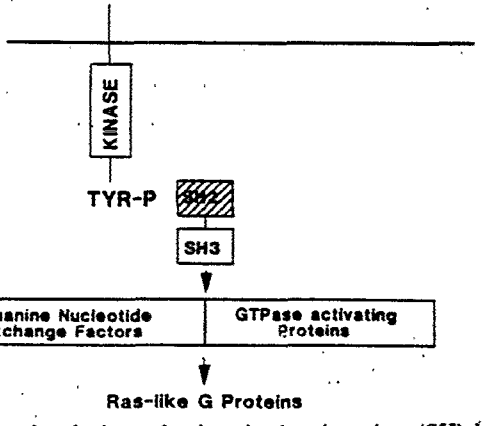

FIG. 2. A postulated schema for the role of *src*-homology (SH) domains 2 and 3 for coupling tyrosine kinases to G proteins. According to this model, proteins with SH2 and SH3 consensus sequences and catalytic domains would be capable of multiple point interactions upstream with tyrosine kinases and downstream with *ras*-like small G proteins (adaptors) and their substrates. Interaction with SH3 domains allows GTPase activating proteins (GAPs) to recruit guanine nucleotide exchange factors regulating *ras* and adaptor proteins (190).

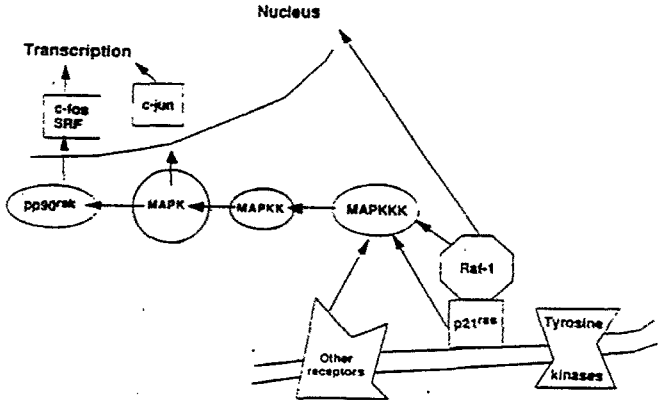

FIG. 3. The role of *ras* in the signaling pathway from tyrosine kinases and other receptors to transcription factors. Raf-1 autokinase is an important station in the *ras*-mediated transduction of the action of many tyrosine kinases— including *src*. It is apparent, however, that the maximal activation of Raf-1 is dependent on synergy between a direct as well as a *ras*-mediated effect. Mitogen-activated protein (MAP) kinases are positioned in the *ras*-pathway leading to the phosphorylation of the ribosomal S6 kinase ($pp90^{rsk}$) and in turn nuclear transcription factors such as *c-fos* and *jun*. MAPK is activated by a cascade of MAPkinase kinase (MAPKK) and MAPkinase kinase kinase (MAPKKK). One of the targets of phosphorylation by EGF treatment of thyroid cells is MAPK (56). The details of this activation pathway are, however, unclear. Platelet dependent growth factor (PDGF), which is expressed aberrantly in anaplastic carcinoma (189), activates the *ras* and Raf-1 pathways (143). Cellular elements intermediary between receptor tyrosine kinase—Sem2 and son of sevenless (sos2) which interact through SH2 and SH3 domains—and *ras* have recently been identified (195). [Adapted with permission from T. M. Roberts: *Nature* 360:534–535,1992 (225). © 1992 Macmillan Magazines, Ltd.]

the conformation of *ras* proteins and are associated with a reduction in GTPase activity (203).

ras Mutations are not sufficient for malignant transformation. The ability of v-Kris-*ras* to transform rat (204) and Ha-*ras* human (205) thyroid cells in primary cultures seemingly contradicts the role of *ras* mutations as early events in thyroid carcinogenesis. Helper viral elements, however, do complement the role of *ras* in transformation in these studies (13). Indeed, even rare events such as double *ras* mutations are not necessarily enough for thyroid cell transformation (206). Moreover, a mutant *ras* gene expressed from a retroviral vector induces thyroid cell proliferation only in the presence of growth factors (207). This growth was inhibited by phorbol esters through regulation of protein kinase C activity (207). Overexpression of the *ras*-21 protooncogene appears to be part of growth-promoting pathways in normal human thyroid and nodular goiter (199).

Distribution of *ras* RFLP in thyroid cancer DNA specimens showed unusual patterns, some of which probably reflected mutation in the coding sequence of the gene (208, 209). An increased abundance of *ras* protein or mRNA was reported in some studies but could not always be related to the degree of tumor de-differentiation (176, 210, 211). Rearrangement of *ras* protooncogenes in thyroid tumors is uncommon and not specific for malignant tumors (179).

ras Gene point mutations are of more interest: mutations in all three families of *ras* oncogenes have been detected in both benign and malignant thyroid tumors (164, 179, 206, 212–223). Most of the mutations were found at residue 61 of H-*ras* and N-*ras* genes (206, 212, 220). Variation in the prevalence of *ras* point mutation thyroid tumors does occur among different series (164, 206, 220) and may be related to the histological and degree of differentiation of the tumors as well as genetic determinants and environmental factors, such as dietary iodide supply (206). It is of interest that thyroid tumors induced by chemical carcinogens in rats involve activation of H-*ras* (219) whereas Ki-*ras* is activated exclusively in 60% of radiation-related tumors in man (224) and rat (219). In the former, radiation does not appear to enhance the overall incidence of *ras* protooncogene mutations (224).

ras Mutations occur predominantly in follicular thyroid cancers (206, 212, 220, 223). Twelve percent of Hurthle cell tumors, a variant of follicular carcinoma, harbored N-*ras* mutations (215). Interestingly, normal tissue adjoining the tumor also exhibited *ras*-activating mutations, emphasizing that cellular events in addition to *ras* mutations are necessary for malignant transformation. The notion that *ras* mutation is relatively specific for microfollicular benign adenomas as opposed to macrofollicular histology (220) could not be sustained (206). An increased frequency of *ras* mutation in metastatic tumors was observed in some (179, 220) but not in other series (206, 214).

The *ras* mutations reported are generally of the transition type (in which a purine is substituted for a purine or a pyrimidine for a pyrimidine), although it has been suggested that transversion mutation (in which a purine is substituted for a pyrimidine and *vice versa*) may be relevant to dedifferentiated thyroid tumors (220). Preferential mutation at H- and N-*ras* 60 is apparently lost in anaplastic carcinoma (220) and awaits further confirmation.

B. c-myc and c-fos

The c-*myc* nuclear proteins act as transcription factors. c-*myc* Dimerizes with its partner *Max* protein and binds to core consensus DNA sequence (226–229). *Max* heterodimerizes preferentially with *myc*, which homodimerizes poorly (230). The formation of *myc*/*Max* dimers is essential for *myc* transforming activity whereas *myc* homodimers are inactive (231). c-*myc* Synthesis declines as the cell cycle progresses (232) and is shut off with inhibition of proliferation associated with differentiation. c-*myc* Has also a central role in some forms of apoptosis (233–235), particularly under growth-limiting conditions. B*cl*-2 mitigates the apoptotic effects of deregulated *myc* expression without affecting its ability to promote continuous cell growth (152, 153).

Alterations in the c-MYC locus occur in a variety of tumors primarily leading to constitutive expression of *myc*. Such deregulation may result in a shift from *Max* homodimers to *myc-Max* heterodimers, and enhances the activation of genes normally modulated during growth and differentiation. Both *myc* and *Max* may be regulated by phosphorylation (236).

Activation of c-*myc* by translocation is restricted to lymphoid tumors. c-*myc* Amplification appears to be a secondary selection factor for increased transformation, as opposed to a primary genetic lesion leading to malignancy (237). The 5′-deletions of the c-*myc* gene described in thyroid tumors (238, 239) were subsequently found to be germline in origin (238) and to exist in unaffected healthy individuals. No gross rearrangements of c-*myc* were documented (214, 240–242). Immunohistochemical studies and mRNA abundance indicated increased expression in thyroid tumor tissue (176, 177, 240, 242, 243). The abundance of c-*myc* transcripts may predict the course of thyroid malignancy: the less differentiated a tumor, the greater the abundance of c-*myc* transcripts (240, 244, 245). Since c-*myc* message abundance was not reflected (at least in papillary carcinoma) in the level of c-*myc* gene products (177), there may be differences in c-*myc* message stability in such tumors. c-*myc* mRNA correlated negatively with that of the TSH receptor (244, 245).

The protooncogenes c-*fos* and c-*jun* are immediate early genes that regulate the expression of specific target genes (246). Depending on cell type the expression of these genes can be activated by second messengers which stimulate protein kinase A and C activities and calcium flux (247–250). The protein products of the genes interact through leucine zipper domains to form *fos*/*jun* heterodimers or *jun*/*jun* homodimers. As components of the AP-1 transcription complex they bind to the regulatory sequence of many genes to initiate transcription (251).

No evidence for rearrangement or amplification of c-*fos* was noted in thyroid tumors (214, 242). Increased c-*fos* transcripts were found in 60% of malignant tumors and 90% of benign adenomas. There was no relationship between the increased transcripts to the biological behavior of the tumors (240). Similar conclusions were reached in our studies of 42

thyroid tumors (our unpublished observations). This pattern of *c-fos* message expression is consistent with its role in differentiation.

C. PTC/RET oncogene

PTC/RET [previously PTC for papillary thyroid carcinoma] oncogene (252–256) results from the fusion of the tyrosine kinase domain of the protooncogene *ret* with a functionally unknown sequence as a result of chromosomal rearrangement. The most frequent partner of this rearrangement is the D10S170 locus (257–259) identified with probe H4 (252). The activation of PTC/RET was first suspected by Fusco *et al.* (260) when they found 25% of papillary carcinoma samples were positive in a transfection assay. The transforming activity was cloned and identified by two independent groups (252, 254). The rearrangement was identified by RFLP and the breakpoint was found to be highly variable within the H_4 sequence but relatively constant in the *ret* protooncogene, mapping to the intron between the transmembrane domain and the first exon of the tyrosine kinase domain (252, 261). All the characterized recombinations, however, produce identical transcripts encoding a protein of 520 residues (252, 257). The activation of RET/PTC results from paracentric inversion of the long arm of chromosome 10, inv (10) (q 11.2 q21) with breakpoint involving the regions where RET and D10S170 are located (262) (Fig. 4).

The unrearranged *ret* protooncogene sequences appear to be involved in neuronal differentiation (263) and to be amplified in neuroendocrine tumors (264, 265). Tyrosine kinase is not activated in the unrearranged *ret* gene product (266), although its natural ligand is unknown. There are two isoforms of *ret* protooncogene which arise by alternative splicing and differ by having 9aa *vs.* 51aa at their respective C termini. The 51aa variant is glycosylated to a variable degree in different cell types (267–270). The *ret* protooncogene is not expressed in thyroid follicular cells but is in parafollicular cells, while its rearranged form is expressed in papillary cancer cells (252).

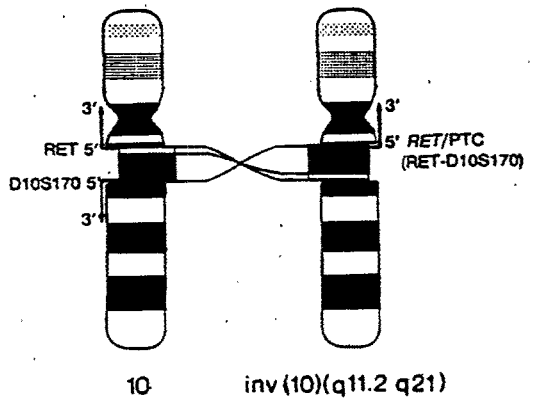

FIG. 4. Paracentric inversion of the long arm of chromosome 10 (10 inv(10)(q11.2 q.21)) in thyroid papillary carcinoma. This inversion places an unknown sequence detected by probe D10S170 upstream from the tyrosine kinase domain of the *ret* protooncogene. [Reproduced with permission from M. A. Pierotti *et al.*: *Proc Natl Acad Sci USA* 89:1616–1620,1992 (262).]

The RET/PTC rearrangement is specific for the thyroid, being undetectable in 250 nonthyroidal malignancies (165, 261, 271). Rearrangement of *ret* previously described in other tumor DNA was found to have occurred *in vitro* during transfection procedures (256). There is a wide geographic variation in the rate of RET/PTC rearrangement in thyroid tumors (Table 1) perhaps related to genetic and/or environmental factors as well as methodology (165, 261, 271, 272). Transfection assays (252, 260), RFLP (252), and reverse transcription-PCR (RT-PCR) (165, 261, 271, 272) have been used to examine oncogene activation and rearrangement. Only the RT-PCR approach is predicated on the identity of the sequence bridging the breakpoint. The rearrangement is apparently specific for papillary carcinoma, although it is unlikely to occur in more than 20% of these tumors (271, 272).

Two groups have, however, reported RET/PTC rearrangement in benign nodules, nodular goiter, and follicular carcinoma (165, 274). In the study of Ishizaka *et al.* (165), protooncogene activation was regionally localized within benign nodules and multinodular goiter and was attributed to the high rate of microscopic papillary carcinoma in Japan. The issue of focal PTC/RET rearrangement in such microfoci may be resolved by *in situ* hybridization with an appropriate probe (275). In a subsequent report, Jhiang *et al.* (261) do not comment on the frequency of PTC/RET rearrangement in follicular adenomas they reported earlier (274). It is interesting that *ret* is rearranged in tumors with follicular/papillary histology (275), giving molecular credence to the designation of these tumors as papillary.

It has been suggested (261) that patients with papillary carcinoma with rearrangement of the *ret* protooncogene have a greater likelihood of developing distant metastases. These studies in a small group await confirmation.

It also bears emphasis that only one third of the samples with *ret* rearrangement show evidence of fusion with H_4 (271). The remaining samples will clearly *not* be identified by an RT-PCR strategy in which a probe spanning the breakpoint is used for screening (261, 271, 272).

The issues of specificity, the mechanisms of RET/PTC activation, and the rearrangement partners other than H_4 are being rapidly clarified (266). *ret* May undergo rearrangements in papillary thyroid carcinoma with a partner other than D10S170 (H4) sequences. This rearrangement may involve either the 9aa (Ptc2) or 51aa (Ptc1) C-terminal form of *ret*. All forms of *ret* rearrangement are distal to the transmembrane domains and therefore localize the hybrid protein intracellularly (Fig. 5). As a consequence, the rearranged gene product constitutively activates the tyrosine kinase, which autophosphorylates at a tyrosine residue. The ptc1 and ptc2 are apparently regulated by phosphotyrosine phosphatases (266). The translocation of the activated rearranged *ret* oncogene may allow them to escape regulatory influences and to constitutively signal mitogenic pathways. It remains to be seen what proportion of papillary carcinoma utilizes non-D10S170 sequences as a partner in the PTC/

TABLE 1. Geographic distribution of PTC rearrangement

	USA[a]	USA[b]	France[a]	Italy[a]	Japan[c]	Saudi Arabia[d]
Papillary	11/65	4/32	8/70	14/42	1/11	1/40
Follicular	0/11	0/3	0/13	0/13		0/4
Anaplastic	0/2		0/5	0/8		0/5
Adenoma		0/8	0/18	0/16	4/19	0/1
Others		0/28			1/2	0/7

[a] Santoro *et al.* (271).
[b] Jhiang *et al.* (261).
[c] Ishizaka *et al.* (165).
[d] Zou *et al.* (272).

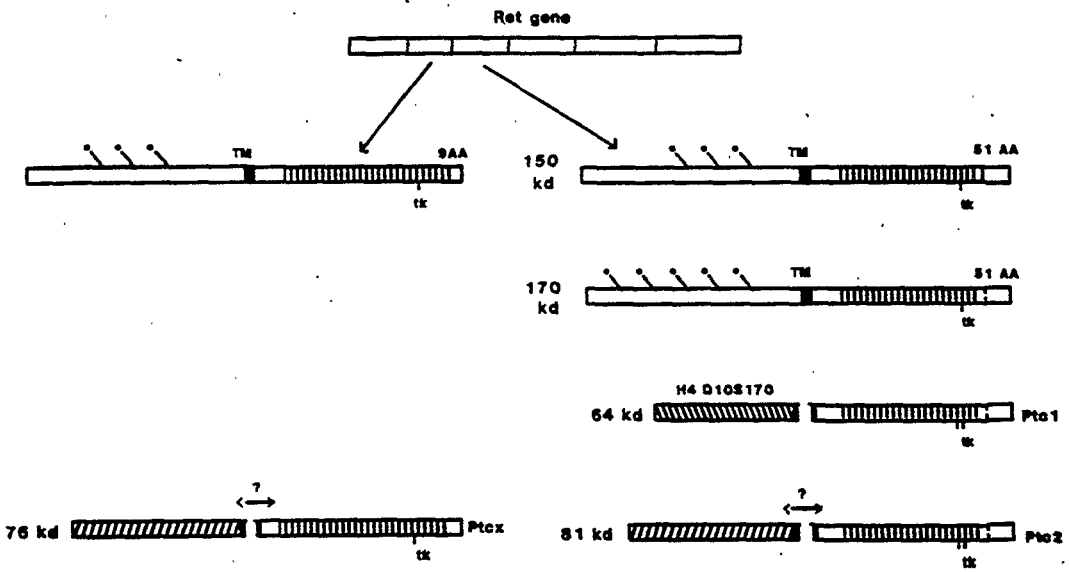

FIG. 5. The rearrangements of the *ret* protooncogene in papillary thyroid carcinoma. The genomic *ret* gene is shown schematically at the *top* of the figure. Two forms of the *ret* protooncogene arise by alternative splicing and have in their COOH termini nine or 51 residues, respectively. The 51 COOH terminus form is glycosylated to variable degrees and the degree of glycosylation may differ from one tissue to another. In the thyroid, 150-kDa and 170-kDa differentially glycosylated forms exist. In thyroid papillary carcinoma, both *ret* protooncogene isoforms are rearranged. Initially, the 51aa. COOH terminus form was found rearranged fusing its kinase domain with an unknown sequence H4 (D10S170) (Ptc1); this probably accounts for no more than 30% of the rearrangement. Other sequences, yet to be identified, can also be involved in the *ret* protooncogene rearrangement and result in an 81 kDa protein (ptc2). Apparently, the short isoform of the *ret* protooncogene undergoes a similar type of rearrangement in papillary carcinoma (shown here as ptcx, a 76 kDa hybrid). All rearranged forms of *ret* protooncogene lose their transmembrane (TM) domain and the fusion proteins are thus localized intracellularly. Whether because they undergo conformational changes or are exposed to new substrates, these fusion products exhibit constitutive activation of the *ret* tyrosine kinase. (⁞ = carbohydrate adducts).

RET rearrangement (266, 271). RET/PTC oncogene-transfected rat thyroid cells lose their differentiated function but are unable to grow in soft agar or to cause tumors in nude mice. An undifferentiated malignant phenotype is obtained when both RET/PTC and *Ha-ras* or *Ki-ras* oncogenes are transfected (276).

We have studied a large number of benign and malignant tumors using the RT-PCR approach (272). Only one out of 40 thyroid papillary carcinomas was found to have undergone PTC/RET rearrangement, suggesting a very low rate of rearrangement RET/D10S 170 locus in the Saudi population (Table 1). Of interest was that this tumor also harbored a mutation in the tumor suppressor gene p53 ($Ala^{161} \rightarrow Thr$). It is unclear whether this is a chance finding or implies selection of *ret* rearrangement for p53 mutation.

Given the activation of *ret* protooncogene by rearrangement in papillary thyroid carcinoma, it is particularly intriguing that missense germ-line mutations of RET have recently been proposed as the genetic basis for MEN2A liability (273).

D. TRK-T1

A second transforming event involving gene rearrangement which activates a tyrosine kinase protooncogene was found in papillary carcinoma (277). The oncogene involved, called TRK-T1, is generated by an intrachromosomal rearrangement that links the tyrosine kinase domain of TRK protooncogene to the 5′-region of the TPR gene, both mapping to chromosome 1 q23-q24 (278, 279). The TRK protooncogene encodes a surface receptor for nerve growth factor (NGF), and the TPR gene a protein with likely cytoskeletal function. The mechanism of TRK-T1 activation is not well understood and may include TRK rearrangement

that activates the gene, moves the hybrid gene product to the cytoplasm exposing it to unusual substrates or may result in conformational changes which mimic those produced by interaction with the ligand, or combinations of these mechanisms (280).

More than one type of rearrangement involving the NGF receptor chain may be generated in papillary thyroid carcinomas (280). The TRK-T1 rearrangement was suggested to be common in papillary carcinoma (277), which, in combination with *ret* rearrangement, accounts for constitutive activation of tyrosine kinase in 50% of these tumors (280).

E. Met

The *met* oncogene is a 190-kDa heterodimer composed of two disulfide-linked (α, β) subunits. The α-subunit (50 kDa) is heavily glycosylated and extracellular. The β-subunit has an extracellular domain involved in ligand binding, a transmembrane segment and cytoplasmic tyrosine kinase domain whose activity is regulated by phosphorylation (281). The mature receptor is derived from glycosylation and extracellular cleavage of a 170-kDa precursor by urokinase (282). The multifunctional cytokine hepatocyte growth factor/scatter factor (HGF/SF) is the ligand for *met*. Multiple *met* transcripts that encode proteins differing in extracellular and cytoplasmic domains have been identified: one form results in uncleaved precursor chain and another in a soluble form (reviewed in Refs. 281 and 283).

HGF/SF is itself a heterodimer arising from the cleavage of a precursor. The light chain is related to serine protease whereas the heavy chain belongs to the plasminogen family of proteins (284–286). HGF/SF binding to *met* enhances receptor kinase activity by tyrosine autophosphorylation of the β- subunits whereas receptor kinase activity is negatively regulated by serine phosphorylation mediated by protein kinase C.

The *met* oncogene is constitutively activated by amplification of the gene or through the expression of a splice variant in which the precursor is not cleaved into the two subunits (281). Activation of *met* is associated with mitogenesis as well as motogenesis and may thus contribute to tumor-aggressive and metastatic behavior in neoplastic human tissues (285, 286). *Met* transcripts and protein were increased in a high proportion of gastrointestinal and hepatocellular carcinomas, and in malignancies arising from meningeal and neural tissues.

Met oncogene is amplified in approximately 70% of papillary and poorly differentiated carcinomas, but in only 25% of follicular carcinomas. *Met* was not detected in anaplastic or medullary carcinomas, nor in a variety of benign thyroid disorders and normal thyroid tissue (287, 288). The amplification of *met* gene was not associated with gene rearrangement, although several samples exhibited an 85-kDa β-subunit soluble variant (288). In most tumors with *met* amplification an autocrine source of HGF/SF is not readily detectable. The elaboration of this ligand by parafollicular cells (289) seemingly establishes a paracrine relationship in the thyroid. Interestingly, of the 10 papillary or poorly differentiated carcinomas also examined for RET and TRK rearrangements, in three RET was rearranged and TRK in one and, except for one tumor with RET rearrangement, all samples exhibited amplification of *met*. This emphasizes the important contribution of *met* in the tyrosine kinase "pathway" for papillary carcinogenesis (288). *Met* overexpression was associated with aggressive clinical and histological phenotype.

Activation of tyrosine kinase, whether by gene amplification or rearrangement, appears to be highly specific for the transformation of thyroid follicular cells into papillary tumors. It is also of interest that papillary thyroid carcinoma is the only type of nonhematopoietic tumor with a high frequency of gene rearrangement.

F. Gsα mutations

The G proteins are a subfamily of the GTP-binding proteins, which include *ras* and *ras*-like proteins (290, 291). The G proteins are heterodimeric, composed of α-, β-, and γ-subunits each encoded by a distinct gene. The α-subunit shows structural and functional homology with other members of the GTP binding protein superfamily. It binds guanine nucleotides with high affinity and specificity and has intrinsic GTPase activity. β- And γ-subunits are noncovalently bound into a dimeric complex. They are necessary for regulating the function of certain α-subunits as well as in directing the trimolecular complex to the plasma membrane (reviewed in Refs. 292 and 293).

G proteins couple a diversity of receptors with their effectors by acting as molecular switches activated and deactivated by the GTPase cycle (292). The variety of G subunits identified, details of the functional domains of Gα, and an overview of the strategies used to elucidate the receptor/G protein coupling were recently reviewed by Speigel *et al.* (292). Gsα is utilized widely as a positive transducer for the activation of adenylate cyclase and calcium channels. Cholera toxin ribosylates Arg^{201} and thus impairs the hydrolysis of the γ-phosphate of GTP. Natural mutations at this residue (*gsp*) have an identical effect. Mutations at residues 232, 233, or 234 (numbering based on 394 residue form of Gsα) have been shown either to inhibit activation of Gα or its GTPase activity. The glutamine at position 227 corresponds to position 61 in *ras* p21.

Activating germline mutations of Gsα may well be lethal. The activating mutations described in McCune-Albright syndrome (294, 295) show tissue mosaicism, suggesting that they have arisen after the first rounds of fertilized ovum division. The Gsα-inactivating mutations noted in Albright's hereditary osteodystrophy may be less deleterious in early development. It is of interest that the activating mutations in McCune-Albright syndrome have been restricted to Arg^{201}, whereas multiple distinct (and heritable) heterozygous inactivating mutations were uncovered in families with Albright's hereditary osteodystrophy (292, 296, 297).

A subset of GH-producing pituitary tumors with high basal adenylate cyclase and which tend to be small (298) have been found to harbor α_s-subunit (*gsp*) mutations (299–301). The mutations involved predominantly $Arg^{201} \rightarrow$ Cys or $Gln^{227} \rightarrow$ Arg or Leu. Similar mutations were described in a

small proportion of thyroid tumors (164, 300, 302, 303). Twenty five percent of follicular adenomas were found to harbor *gsp* mutations (298, 302, 303). There may be a predilection for these mutations in microfollicular adenomas. The rate of these mutations was found in a recent study to be less than 3% (304). Some of the mutations were novel, *e.g.* $Gln^{227} \rightarrow$ His. Mutations reported in papillary and follicular carcinoma are uncommon (300, 302, 304); the substitutions were also unusual ($Arg^{201} \rightarrow$ ser, $Gln^{227} \rightarrow$ Lys and $Gln^{227} \rightarrow$ His) (302). As anticipated, *gsp* mutations were more common in tumors selected with high basal adenylate cyclase activity (302).

A recent study (164) is worthy of separate consideration, since it shows geographic variation (Germany *vs.* the United States) in the rates of *gsp* and *ras* oncogene activation in 32 differentiated carcinomas and regional differences in the activation of these two oncogenes in fragments of the same tumors. The authors suggest that in samples from iodide-deficient Germany *gsp* mutations are more common than *ras* (in direct contrast to US samples) and that these mutations are much more frequently identified in tumor section than from whole tumors. Because thyroid tumors are monoclonal (see above) some of these mutations are probably late events. They may, nevertheless, modify tumor biological behavior (164). The potential interaction between *ras* and *gsp* mutation in thyroid tumor requires further clarification.

To what extent regional variation within the same tumor accounts for variation in *gsp* mutation within iodide-sufficient regions is unclear (298, 302–304) and, indeed, given the number of samples studied, the differences between some of the studies are not statistically significant. Our own experience with a detailed study of 32 thyroid tumors uncovered no mutation at Arg^{201} or Gln^{227}. We have, however, found a mutation in exon 1 of Gsα ($Lys^{34} \rightarrow$ Arg) in one follicular adenoma. The mutation is placed in a functional attenuator domain in Gsα. These experiments were predicated on the notion that N-terminal domains of Gsα regulate the rate of αs activation by guanine nucleotides independent of GTPase activity intrinsic to Gsα. It is likely a modulator domain of GDP dissociation and GTP activation (305). Whether this is a random somatic mutation or a normal polymorphism, and whether it alters Gsα function and is thus relevant to thyroid tumorigenesis, remains to be determined.

The potential oncogenic role of constitutive elevation of cAMP is emphasized by transfection of A_2 adenosine receptor cDNA into cells or its targeting for expression in the thyroid of transgenic mice under the influence of the thyroglobulin promotor (306, 307). In the latter case, constant adenylal cyclase activation is associated with thyroidal hyperplasia and nodular goiter (307). On the other hand, $Gi_\alpha 2$, a Gi_α subtype regulated by TSH (308), was found to be constitutively increased in autonomous adenomas, but not in thyroid cancers. The increase in $Gi_\alpha 2$ was associated with decreased cAMP levels in tissues tested (308).

IX. The TSH Receptor

The TSH receptor is a member of the seven-transmembrane segment G protein-associated receptors. In common with the LH/CG and FSH receptors, the TSH receptor has a large extracellular domain with multiple glycosylation sites (see Ref. 309 for review). Structure-function analysis of the receptor has delineated some of the receptor functional domains (310, 311). The second cytoplasmic loop, COOH-terminal domain of the third cytoplasmic part, and amino terminal of the intracellular tail appear to be important in transducing the signal initiated by agonist binding (309, 310). The intracellular tail does not possess a consensus sequence for protein kinase A substrate but does have several potential kinase C substrate sequences (312). The TSH receptor third intracellular loop has a distinctive motif found in nonreceptor tyrosine kinases (312). Mutation of the tyrosine in this motif did not, however, alter receptor activity (310). Three major transcripts (4.5, 1.7, 1.3 Kb) were observed, probably resulting from alternate splicing of the mRNA. The two smaller variants can potentially result in soluble TSH receptor forms (313, 314). Two insertions (8aa at the NH_2 and 50aa at the COOH-end of the extracellular domain), are characteristic of the TSH receptor (see Ref. 309). The former is essential for TSH binding and receptor activation, whereas the biological relevance of the latter is unclear.

It is now possible to verify, at the molecular level, earlier observations of negative correlation between the degree of differentiation of thyroid tumors and their ability to bind [^{125}I]TSH and to stimulate adenylate cyclase (reviewed in Ref. 315). Benign thyroid tumors have a similar number of TSH receptor transcripts to those found in normal thyroid (244, 245). With decreasing degrees of differentiation, fewer TSH receptor transcripts are found. Indeed, a tolerable degree of negative correlation between the clinical staging of the disease and receptor mRNA abundance exists (244, 245), with some exceptions (244). With an increase in dedifferentiation, however, expression of thyroperoxidase is lost (245, 316) well before thyroglobulin transcripts (245). The loss of TSH receptor gene expression appears to be a late event in thyroid tumor dedifferentiation (245). We did not find a preferential expression of receptor variants in thyroid tumors (244). The relevance of a report of variation in the size of TSH receptor gene determined by RFLP in follicular adenomas (317) is unclear.

Increasing dedifferentiation of thyroid tumors and decrement in TSH receptor mRNA was associated with an increase in β-adrenergic receptor transcript numbers, deduced by quantitative PCR (318). It is unclear whether these mRNAs are translated into functional β-adrenergic receptors or whether they have undergone mutations that will render them constitutively activated. This area of investigation is worthy of further exploration, as site-specific mutations of the C-terminal part of the third intracellular domain of the α_{1B}-adrenergic receptor was associated with constitutive activation of PI hydrolysis (319) and neoplastic transformation. These mutants have not, however, been observed naturally.

That spontaneous mutations in the third intracellular loop of the TSH receptor are relevant to thyroid cell transformation has only been recently demonstrated. Thus Parma *et al.* (319a) found mutations in three of 11 autonomous (benign) thyroid nodules. One of these mutations was found at posi-

tion 623 (Ala → Ile) and the other two were found at position 619 (Asp → Gly). The mutations were associated with constitutively activated basal cyclase activity. Since screening has been linked to the third intracellular loop it is possible that more complete screening would locate mutations in other parts of the receptor (319b).

X. Tumor Suppressor Genes

The existence of tumor suppressor genes derived from three lines of evidence: 1) somatic cell hybridization of tumor with normal cells (320–323), 2) the realization of Knudson's "two hit" postulate (324–326) in familial retinoblastoma (324), and 3) arising from the retinoblastoma paradigm (327–333), the correlation of loss of chromosomal segment heterozygosity with inactivation of putative tumor suppressor genes. The use of anonymous DNA markers has facilitated the search for the tumor loss of heterozygosity (LOH), since chromosomal loss extends to regions flanking suppressor genes (334, 335). Tumor suppressor genes may also be identified by difference cloning or differential display of mRNA (336, 337).

A. LOH in thyroid tumors

Studies have been undertaken to explore chromosomal structural and numerical abnormalities in thyroid tumors. The number of patients studied is, however, limited and has incriminated a variety of chromosomal regions (125; and Ref. 338 for review). Many may be secondary to thyroid cell transformation (338).

LOH involving chromosome 3p was implicated in and specific to follicular carcinoma (338). 3p LOH was described in small cell lung carcinoma (339), non-small cell lung carcinoma (340, 341), and breast and testicular tumors (342–344). Renal cell carcinoma harbors a more proximal deletion (3p14–21) (345) than small cell lung carcinoma (p21–23). It is intriguing that Matsuo *et al.* (346) reported LOH at 11q13 in approximately 14% of follicular adenomas. It is implied that some follicular adenomas will pursue a line of progression distinct from that in line to follicular carcinomas and thus subvert the assumption (3, 338) that follicular adenomas are intermediate between normal follicular cells and follicular carcinomas. Moreover, the chromosomal region implicated (346) harbors the multiple endocrine neoplasia I gene (347–349) as well as oncogenes and growth factor genes. The findings of LOH in thyroid tumors, therefore, bear confirmation in larger series of patients.

B. Retinoblastoma (Rb) gene

The Rb gene spans 180 Kb and 27 exons and maps to 13q14 (326, 350, 351). It encodes a 110-kDa nuclear phosphoprotein (350, 352). Rb binds the viral oncoproteins SV40 large T antigen, human adenovirus EA1, and papillovirus E7 (353–357). This binding inactivates Rb's growth suppressing function (358). All the viral oncoproteins bind to an underphosphorylated form of Rb (359).

The Rb protein switches between a hyperphosphorylated and relatively underphosphorylated state in a cell cycle-specific manner (360–364). By binding them, pRb regulates the actions of the transcription factor E2F (365–370), and cyclins D_1 and D_3 (371, 372) which play critical roles in promoting cell cycle progression and parallel the already defined role of p107 (370–375). Seemingly, these factors bind in the same pRb pocket as do oncoproteins. Cyclin D_2, which accumulates in a cell cycle-dependent manner, is instrumental (in association with cd kinase 2) in the hyperphosphorylation of pRB and thus its release of cyclin D_1 and D_3 (372). This schema explains the negative regulation of *myc* expression by Rb/E2F complex and the observation that *myc* can overcome the ability of Rb but not p53 in affecting the G_1 phase arrest (376, 377). It also raises the possibility that mutations in or amplification of E2F or of specific cyclins may result in a phenotype similar to a loss of Rb (370, 371).

A subtle role of Rb in growth-inhibiting pathways is compatible with normal embryogenesis in familial retinoblastoma, where a defective gene is passed on in the germline and with the normal development of mice in which one allele has been inactivated (378–380). Mouse embryos in which both alleles have been disabled, however, fail to reach term and show abnormalities in neural and hematopoietic development (378–381).

Somatic Rb gene inactivation has now been implicated in a number of tumors (382), aside from retinoblastoma and sarcomas which are seen in relation to germline heterozygosity at the Rb locus (383, 384). The range of tumors involved is, however, likely to be more restricted than for mutations in p53. These include cervical carcinoma [whether or not they are positive for human papilloma virus sequences (385)], small cell and non-small cell lung carcinomas, bladder, and breast carcinomas (386–394). Some of these tumors also harbor p53 mutations (385).

The mechanisms that inactivate the Rb gene include deletions, insertions, or point mutations and have been uncovered in retinoblastomas, osteosarcoma, and soft tissue sarcoma which develop later in life in patients with retinoblastoma (350, 395, 396) as well as in a variety of tumors and tumor cell lines (382). Mutations that affect serine and threonine phosphorylation of Rb protein or its ability to bind oncoproteins have been mapped to two domains: codons 393–572 and codons 646–772 spread over exons 13–22 (397–399). The frequent deletions of exons 13–17 in retinoblastoma suggest that they may contain recombinational "hot spots" (351). Exonic deletions of the Rb gene are usually accomplished by mutation at splice junction resulting in in-frame deletions (382, 385). Genomic DNA from such tumors may not exhibit obvious abnormalities (389, 400). These mutations result in lack of gene expression or a defective Rb protein (382, 401).

The development of thyroid tumors in transgenic mice in which SV40 T antigen was targeted to the thyroid gland under the influence of thyroglobulin promotor (402) led us to study Rb gene mutations in thyroid tumors. Mutant Rb alleles were found in 55% of thyroid carcinomas but in none of the benign tumors. The rates of Rb mutations were similar in anaplastic and differentiated thyroid carcinoma. The mutations were either attributable to abnormal Rb mRNA splic-

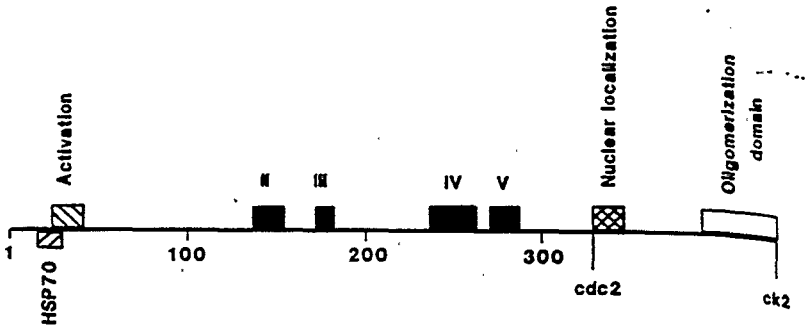

FIG. 6. Functional domains of the p53 protein. The activation domain of p53 has been mapped to the NH_2-end of the protein (residues 20–42). The heat shock protein (hsp) 70 binding domain is further upstream and overlaps the activation domain. The oligomerization domain (residues 344–393) is at the COOH end of p53, whereas the sequence necessary for nuclear localization is mapped to residues 316–325. Domains II-IV represent areas that are highly conserved among species. Some 98% of all p53 mutations described in tumors occur in these four domains (also see Fig. 7). cdc2 Represents serine 315 phosphorylated by $p34^{cdc2}$ kinase; CK2, serine 392 phosphorylated by casein kinase 2. [Adapted with permission from B. Vogelstein and K. W. Kinzler: *Cell* 70:523–526,1992 (162). ©1992 by Cell Press.]

ing, C-terminal deletion, or point mutations (403). The most common mutations were deletions of exon 21, resulting in the fusion of exons 20–22. The deletion was, however, not present in genomic Rb gene sequences indicating that the deletion was the result of abnormal Rb mRNA splicing. One papillary carcinoma sample contained an in-frame deletion of most of exons 19 and 21 as well as the entire exon 20. The same sample also contained a deletion of exon 21. Twelve percent of malignant tumors harbored both Rb and p53 mutations. p53 And Rb double mutations appear to be more frequent in advanced disease stage. Thus Rb may be an important factor in thyroid cell malignant transformation, and possibly in tumor progression.

C. p53

The normal gene for p53 encodes a 393-residue long nuclear phosphoprotein mapping to chromosome 17p13 and spanning 11 exons. Exon 1 does not encode protein sequences (404). Cross-species comparison reveals five highly conserved domains, four of which fall within exons 5–8 (404): codons 117–142 (domain II); codons 171–181 (domain III); codons 234–258 (domain IV), and codons 270–286 (domain V). The C-terminal region of p53 was predicted to form an amphipathic helix-like structure (405, 406). p53 Oligomerizes through its C terminus to form tetramers (407). Its NH_2-terminus regulates the expression of downstream genes, which negatively control growth (408–412). The heat shock protein (hsp) 70 binding region (413) in the NH_2-terminus overlaps the activation domain. hsp 70 May act as a chaperone that facilitates oligomerization. The major nuclear localization signals are mapped to codons 316–325 (414) and include the target of serine phosphorylation by p34 *cd* kinase 2 (415). As most mutations impair the ability of p53 to bind DNA and/or result in dysfunction of the NH_2-terminus activation domains, they must affect the conformation of the entire protein (162, 416–418) (Fig. 6). Wild type p53, but none of the mutants tested, transactivates the putative promotor sequences (408). p53 Inhibits transcription from minimal promotors or it binds to TATA-binding protein, thus inhibiting transcription indirectly (418–420).

p53 Appears to regulate growth-inhibiting signals at the G1-S transition of the cell cycle and monitor the genetic integrity at cell division in the same way as the *rad 9* gene in yeast (421). The effects of p53 are apparent only in stressed cells (162, 163, 422). Normal cells exposed to radiation (156), UV light, or UV-mimetic drugs (423) exhibit an increase in p53 expression and are arrested in G_1 until repair is effected. In contrast, cells harboring mutant p53 genes continue to divide and either die or accumulate genetic defects leading to tumorigenesis (157). In certain tumor systems, the prior activation of *myc* cooperates with *ras* in neoplastic transformation in the absence of p53 mutations, whereas in the absence of an activated gene there is a strong selection for p53 mutation by *myc* resulting in hyperplasia and not neoplasia (151, 424) and stresses the role of p53 in apoptosis (151).

p53 Was initially found through its association with SV40 large T antigen oncoprotein. Considered (425) an oncogene which cooperates, like *myc*, with *ras* in transforming cells (426, 427), it was later shown to be a tumor suppressor (147, 428). The p53 genes employed in those early experiments were inactivated by mutations (429). Like the Rb protein, p53 binds SV40 large T antigen, the E6 proteins of human papilloma virus, and EA1 of adenoviruses (425, 430–433). These oncogenic viruses sequester the p53 protein by binding it with p53 protein.

Germline p53 mutations are found in Li-Fraumeni syndrome (421), a familial cancer syndrome in which relatives develop diverse malignancies including breast carcinoma, sarcomas, and brain tumors (434, 435). Germline mutations have now been found in sporadic patients with cancers comprising Li-Fraumeni syndrome, specifically those with second tumors (436–439) and in patients with familial breast cancer (440, 441). p53 Mutations in Li-Fraumeni syndrome are widely distributed among the p53 genes between amino acid residues 72 and 325 (418, 421, 442, 443).

Somatic p53 mutations have been described in a wide variety of human cancers (428, 444–451). Some 98% of these mutations are found in that part of the molecule encoded by

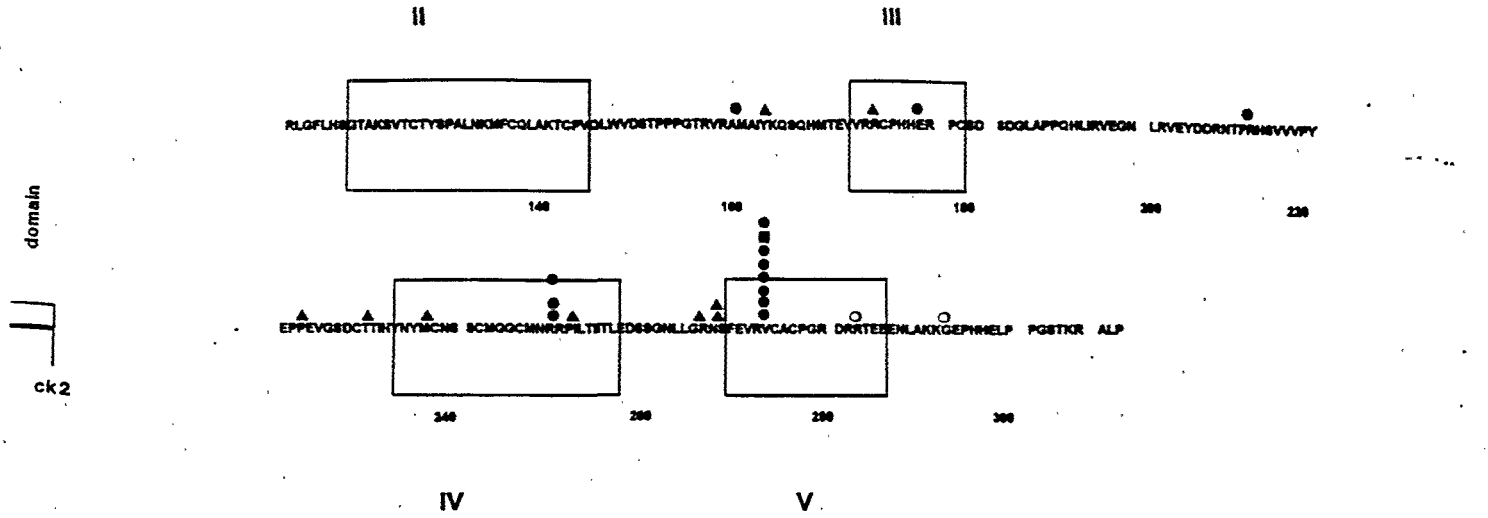

FIG. 7. Distribution of p53 mutations described in malignant thyroid tumors and in thyroid tumor lines. (▲, well differentiated tumors; ●, anaplastic tumors; ■, anaplastic cell lines; O, well differentiated cell lines). II-IV represent domains highly conserved among species studied. Domain II includes residues 117–142; domain III, residues 171–181; domain IV, residues 234–258; and domain V, 270–286, the residues are numbered according to Soussi *et al.* (404). *Single letter* residue abbreviations are as follows: A, Ala; C, Cys, D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, leu; M, Met; N, Asn; P, Pro; Q, Glu; R, Arg; S, Ser; T, Thr; V, Val; W, TRP and Y, Try. Some 98% of all the mutations described in a variety of malignant tumors have been mapped to these four domains. By contrast, only 65% of the mutations described in malignant thyroid tumors or tumor lines map to these domains. Eleven of 13 mutations described in anaplastic tumors do map to domains III, IV, and V. Position 273 in homology domain V and 248 in domain IV appear to be mutation hot spots for anaplastic thyroid tumors and may be involved in tumor progression. It is highly unlikely that mutations outside the evolutionary conserved regions found in well differentiated tumors are rare polymorphisms.

exons 5–8 (428, 444). The missense mutations are so numerous that some are bound to represent previously undetected rare polymorphisms or may not affect p53 function (452–454). Mutated p53 proteins may bind DNA abnormally (408, 415), may exhibit NH_2-termini (*e.g.* mutants at codons 143, 175, 248, and 273) which are unable to activate reporter genes (408), may bind tightly to hsp 70 (mutants at codons 135, 175 but not 273) and to each other forming stable complexes and prolonging the mutant's half-life (429, 455), or may fail to bind to conformation-dependent antibodies (456) (mutants at codon 135, 175) or to suppress growth or transformation in transfection assays (162, 428). Three codons, 175, 248, and 273, appear to be particularly targeted for mutations (428).

When mutations are examined by tumor type, some differences emerge with respect to the position of the hot spots, frequency of mutation involving transitions compared to transversion (444). This exercise has yielded some site-specific information regarding the mutagens involved. Thus hepatocellular carcinoma in areas with high exposure to aflatoxin B1 ingestion and hepatitis B virus is associated predominantly with codon 249 mutation (457, 458). Likewise in human squamous cell carcinoma of the skin, p53 was mutated exclusively at pyrimidines, with frequent CC → TT double base changes, pathognomonic of UV light effect (459).

Loss of one allele with mutations in the remaining allele is characteristic of p53 inactivation (428). Unlike Rb, however, mutation at one p53 allele may also contribute to human carcinogenesis through negative dominant effects. Indeed, some mutant p53 proteins found in human tumors can cooperate with the *ras* oncogene to transform primary cells, suggesting that they can inactivate endogenous wild-type p53 proteins (428). p53 Mutants differ in dominant negative effect, *e.g.* Mutants at codon 175 are severalfold more efficient than codon 273 mutants in cooperating with *ras* (428, 455). Wild-type p53 may also be bound and sequestered by E6 oncoprotein in human cervical carcinoma or the product of the MDM2 gene (162) found to be amplified in a high proportion of human sarcomas (459–461).

Mutations of the p53 gene in human neoplasia are generally regarded as late events (9, 462). There is, however, mounting evidence to show that it can also occur early in the neoplastic sequence in some malignancies (423, 446, 463, 464).

Mutations in the p53 gene appear to be relevant to the development and progression of malignant thyroid tumor. Initially, no mutations of p53 were detected in 129 thyroid tumor samples studied by immunohistochemistry or 20 tumor samples (465) by genomic DNA sequencing of amplified exons 5, 7, and 8. Both a cell line established from recurrent follicular carcinoma and the original tumor harbored a homozygous transitional (CGT → CAT) mutation at codon 273 (465). Concentration of mutations in exon 6 and/or gross contamination of tumor with nontumorous tissue has been invoked to account for the negative results in that study (416, 466).

Three other studies have documented p53 mutations in thyroid carcinoma (416, 466, 467). In two the mutations were predominantly or exclusively found in anaplastic tumors (466, 467), whereas in the third mutations were found in well differentiated malignant thyroid tumors (416). The geo-

TABLE 2. p53 Mutations in thyroid cancer

	Type	Stage	Age	Sex	Exon	Codon	Base change	Amino acid change
1.	AC	IV	46	M	5	159	GCC → CCG	Ala → Pro
						177	CCC → CC	1 bp deletion (frame shift)
2.	PC	IV	70	F	8	268	AAC → AGC	Asn → Ser
3.	FC	II	48	F	8	292	AAA → AGA	Lys → Arg
4.	PC	II	46	M	7	256	ACA → ACG	No
5.	PC	II	45	M	7	250	CCC → CTC	Pro → Leu
6.	PC	II	24	F	6	212	TTT → TTTT	1 bp addition (frame shift)
					8	282	CGG → TGG	Arg → Trp
7.	PC	I	23	F	8	283	CGC → CGT	No
8.	PC	II	25	F	7	231	ACC → GCC	Thr → Ala
9.	PC	I	32	F	6	222	GAG → GAA	No
10.	PC	I	36	F	8	268	AAC → CAC	Asn → His
11.	PC	I	26	F	7	237	ATG → ATA	Met → Ile
12.	PC	II	27	F	5	161	GCC →BCC	Ala → Thr

Review of histology of samples showed that samples 2, 7, and 8 had evidence of solid foci suggestive of dedifferentiation. AC, Anaplastic carcinoma; PC, papillary carcinoma; FC, follicular carcinoma.

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graphic variation in the apparent role of p53 as a factor in tumor progression in the United States and Japan, and in tumor development in Saudi Arabia, is intriguing and may be related to genetic or environmental factors. Certainly, all three studies were reported from apparently iodide-sufficient areas. Scrutiny of the locations and nature of the mutations involved, most of which are transitional in nature, does not provide clues to potential environmental mutagens. In the studies by Ito *et al.* (467) and Zou *et al.* (416), the mutations appear to be relatively scattered in the p53 gene (Fig. 7). That all five p53 mutations detected among six anaplastic carcinoma samples showed a CGT → CAT (Arg → His) transition at codon 273 in one study (466) is intriguing. Ninety percent of the point mutations in the anaplastic thyroid tumors and 50% in the well differentiated carcinoma (416) were G:C→A:T transitions. Positions 273 (466, 467) and 248 (467) (cpG dinucleotides) appear to be mutational hot spots in anaplastic thyroid carcinoma. Methylated cytosines occur exclusively at CpG dinucleotides and are a major site of spontaneous and probably enzyme-catalyzed deamination driven mutations (468, 469) of the p53 gene (468). The possibility that these mutational hot spots are preferen-

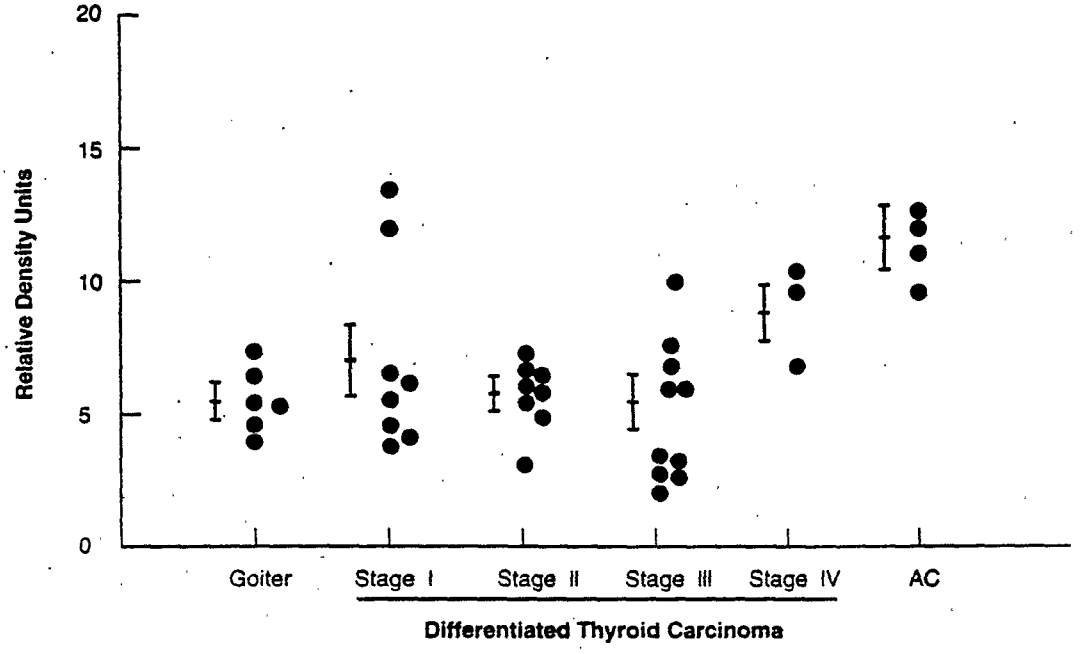

FIG. 8. The distribution of nm23 transcripts in thyroid tumors. There was no difference between benign nodular thyroid tissue samples (goiter), stages I through III of thyroid carcinoma. Samples from patients with anaplastic tumors and those at stage IV were significantly higher. These results suggest no correlation between the tumors' metastatic potential and nm23 transcript abundance. As close correspondence between transcript abundance and nm23 protein expression was described (507), the results cannot be explained away by variation in message stability. Thyroid tumors were staged on the basis of the TNM international classification (see Ref. 508). AC, Anaplastic carcinoma.

TABLE 3. Oncogene and tumor suppressor genes incriminated in thyroid tumors

Oncogene/antioncogene	Tumor type	Frequency (%)	Lesion	Environment
Ras	Adenomas	80	(H-ras 61) Point mutation	Iodide deficient
	Follicular carcinoma	50	(H-ras 61) Point mutation	Iodide deficient
	Adenomas	17	(H-ras 61) Point mutation	Iodide sufficient
	Follicular carcinoma	10	(H-ras 61) Point mutation	Iodide sufficient
	Anaplastic carcinoma	60	Point mutation	?
	Thyroid tumors[a]	60	(K-ras) Point mutation	Radiation
PTC/ret TPC	Papillary carcinoma	25	Rearrangement	?
Trk	Papillary carcinoma	10	Rearrangement	?
	Multinodular Goiters	5		
Met	Follicular carcinoma	22		
	Papillary carcinoma	74	Increased expression	?
	Poorly differentiated carcinoma	75		
c-*myc*	Malignant tumor[b]	57	Increased expression	?
	Papillary carcinoma	30		
	Adenoma	70		
c-*fos*	Malignant tumor[b]	60	Increased expression	
	Adenoma	90		
gsp	Differentiated carcinoma[c]	10	Point mutation	Iodide deficient
	Adenoma	3–25	Point mutation	
	Multinodular Goiter	5	Point mutation	
p53	Differentiated carcinoma[c]	25	Point mutation or	
	Anaplastic carcinoma	86	Deletion or insertion	?
RB	Differentiated carcinoma[c]	54.5	Deletion or	
	Anaplastic carcinoma	60	Mutation	?

[a] Thyroid tumors: papillary, follicular, anaplastic, and adenoma.
[b] Malignant tumors: papillary, follicular, and anaplastic carcinoma.
[c] Differentiated carcinoma: papillary and follicular carcinoma.

tially used in different races or geographic locations requires further exploration (466, 467).

Eight of the 25 mutations including two in anaplastic thyroid carcinomas were outside the homology domains II-V (Fig. 7). As the mutations in well differentiated carcinomas do not coincide with any of the three polymorphisms described (453, 454, 470, 471) nor with nondeleterious mutation at codon 181 (452) and are restricted to malignant tumors (416), it is likely that they are causally related to malignant thyroid cell transformation. Their deleterious nature can, however, be definitively assessed by transfection assays.

Interestingly, all 20 mutations reported from tumor tissue involved only one p53 allele and cannot be attributed to contamination with normal thyroid tissues. By contrast, three of four thyroid carcinoma cell lines (465, 466) showed homozygous inactivation of p53, suggesting that this attribute may confer on thyroid epithelial cells the ability to grow in culture. Our data (416) suggest that the exon 5–8 domains of p53 are mutable in malignant thyroid tissue as well as the subject of frame shifts (Table 2). The reasons for these mutation pressures would be interesting to explore.

XI. Progression to Metastatic Potential

Two general and overlapping mechanisms may lead to the acquisition by cancer cells of metastatic potential: 1) malignant cells with a growth advantage in the primary lesions are more likely to metastasize (472); 2) cancer cells that metastasize acquire additional properties as a result of activation or inactivation of specific genes (473).

With stepwise accumulation of cellular events, malignant cells elaborate their own growth factors, become resistant to cytokines and growth factors that are normally inhibitory to normal cell growth, and may actually grow under the influence of these inhibitory factors. Primary tumors are thus clonally dominated by subpopulations of malignant cells with metastatic competence (474, 475). Since these cells frequently aberrantly manufacture their own growth factors and cytokines they develop a private autocrine loop that allows for multigrowth factor independence (476–478). Early in this process, the cells become growth factor competent by elaborating ectopically (479, 480) basic FGF (bFGF), NGF, PDGF, and IGF-1 (481, 482). Further malignant progression

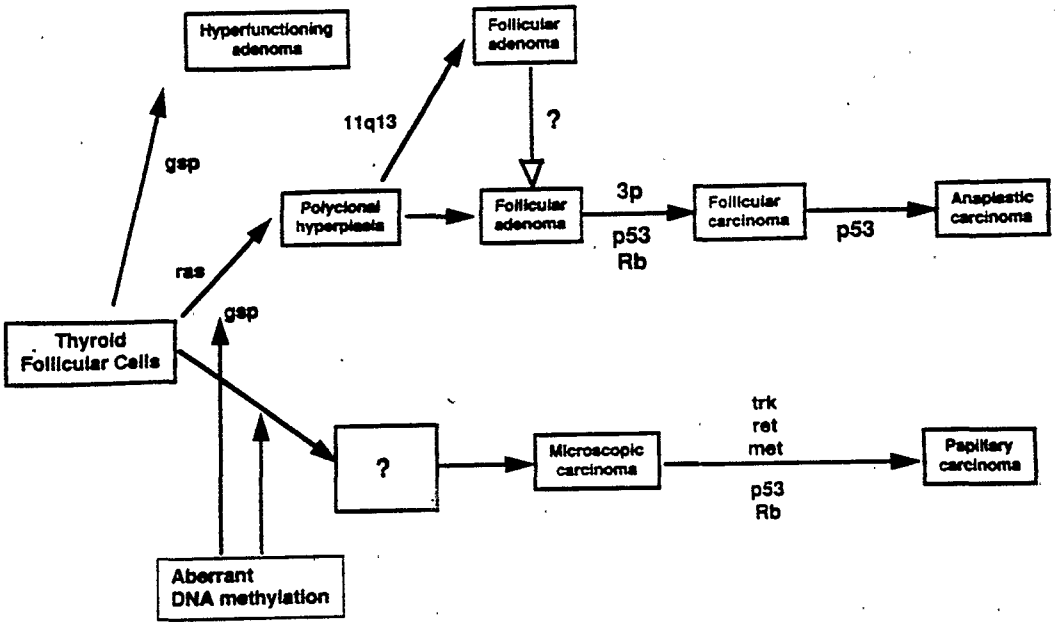

FIG. 9. Current understanding of molecular events in thyroid carcinogenesis. The figure synthesizes proven and putative events as well as others of a speculative nature. The pathways leading to follicular or papillary carcinoma are divergent. The point(s) at which additional events select one of polyclonally proliferating cells for monoclonal growth and local dominance is unclear, and we do not know that hyperplasia is a prerequisite for microscopic papillary carcinoma foci. We are limited not only by lack of information at the precancerous stage but also by the small numbers of follicular carcinomas studied in most series. It is clear that *ras* oncogene activation is specific for the follicular carcinoma route and the rearrangement of *ret*-protooncogene and TRK1 protein tyrosine kinases for the papillary carcinoma fork. Seemingly, loss of heterozygosity (LOH) at chromosome 3p is specific for follicular carcinoma and implies the involvement of a tumor suppressor gene mapping to that chromosomal region. LOH at 11q13 may well be one of several mechanisms for clonal appearance of follicular adenomas. Because of the generally held role of tumor suppressor in tumor progression, we speculate that these adenomas have a high likelihood of becoming malignant. p53 And Rb are assigned roles in malignant transformation of thyroid cells to papillary and probably follicular carcinoma and progression to anaplasia from follicular carcinoma. A role for *gsp* in follicular adenoma formation is secure. It is, however, possible that *gsp* mutations appear late in malignant thyroid tumor and perhaps determine tumor behavior.

in some epithelial cancers is associated with their transition from growth inhibition, *e.g.* by TGFβ and IL-6 to growth stimulation by these factors (483–485). Sustenance of these highly malignant (and metastatic) cells by IL-6 has now been demonstrated in a variety of epithelial cancers (486–491). The relationship of the discrete multiple genetic events leading to malignancy to the increasing growth independence and subversion of inhibitory to stimulatory signals remains to be elucidated, as is the notion that resistance to one cytokine selects for resistance to other related or unrelated cytokines (472).

Given that beyond the size of 2–3 mm, solid tumors require their own blood supply, neurovascularization is a necessity for tumor success. These vessels also provide a means whereby cells with a high growth potential metastasize. Angiogenesis-promoting proteins synthesized by tumors include bFGF, EGF, vascular-endothelial growth factor, hepatocyte growth factor, pleitrophin, and PDGF (285, 492, 493). The degree of angiogenesis determines tumor progress and its outcome (494, 495).

XII. nm23

A candidate for a specific gene product which influences metastatic potential of tumors is nm23 (473, 496). Nm23 was identified on the basis of its reduced steady state mRNA levels in several tumor cell lines with high metastatic potential (497). The gene (NME1) (498) was later found to be highly homologous with *Drosophila* abnormal wing disc (awd) developmental gene (499) and with the nucleoside diphosphate (NDP) kinases (500–502). A second gene (nm23-H2) which is 88% identical to nm23-H1 has been described (503). The two isoforms of nm23, which map to 17q 21.3 (498, 504), correspond to A and B chains of NDP kinase (504, 505) and are assembled in variable combinations into hexamers (504).

The abundance of nm23 mRNA and protein showed a negative correlation with metastatic potential in experimental melanomas (497) and human breast cancer (506, 507). Furthermore, transfection of the nm23 gene into melanoma cells reduced their metastatic potential (509). Deletions or mutations of nm23 were found to correlate with metastatic behavior of tumors (510–512). The correlation of nm23 transcript number with metastases was, however, not noted in colonic cancer (513), neuroblastoma (514), and in some solid tumors (515).

We have studied the abundance of nm23 in 49 thyroid tumors with a wide range of histology. Our results indicate that nm23 transcript abundance did not negatively correlate with metastatic potential or with clinical staging of the disease in general. The highest level of nm23 transcripts was found in anaplastic carcinoma (Fig. 8). We have also used

single strand conformational polymorphism to explore the possibility that the nm23 gene may harbor somatic mutations and/or deletions in thyroid carcinoma. None were found. Lack of mutation was further confirmed by sequencing nm23 cDNA from four tumors, two with normal level expression and two with high expression (516).

The nm23 data should be interpreted in light of the identity of two nm23 gene products with the NDP kinase A and B chains (504, 505). The assembly of the two chains with different sets of intra- and intermolecular disulfide bonds and/or ratios of A and B chains could yield a number of isotypes. It appears that the A chain decreases much more than the B chain in metastatic tumors (507) and may thus preclude the formation of specific complexes in which its participation is critical. A more extreme example would be loss of copies of NME1 and NME2 by loss of heterozygosity. It, of course, remains to be seen as to which NDP kinase complex is actually involved in limiting metastases. This proposed mechanism would not, however, account for the increase in nm23 with dedifferentiation in some tumors.

NDP kinases may be responsible for high GTP concentrations in the proximity of G proteins (517). The suggestion that NDP kinase activates the small GTP binding protein, ADP ribosylation factor (518), which is necessary for nonclathrin coatemers, could not be supported (519, 520). Apparently Golgi membranes can specifically catalyze the exchange onto ADP ribosylation factor (519, 520). The mechanism whereby specific or all NDP kinases inhibit metastatic behavior is unclear, but is likely due to effects downstream of these gene products. That NDP kinase suppresses differentiation of leukemic cells in the mouse (521) suggested a role for nm23 in proliferation and differentiation. The recent recognition that nm23-H2 (but not nm23-H1) is a transcription factor for *myc* is intriguing (522).

XIII. Perspective

One aim of cancer research is to understand the etiological basis for carcinogenesis, with the long term objective of reducing cancer incidence. Epidemiological studies may incriminate environmental factors as well as genetic predisposition in the etiology of cancer. The recognition that cancer is a multistep process and the rapid elucidation of these steps may allow targeted prophylaxis and therapy. The picture in thyroid cancer is emerging at a modest pace compared to other cancers (Table 3). We have thus drawn from experiences with other cancers in this respect.

A number of cellular steps are necessary for thyroid cell transformation. These result in the delivery of constitutive and/or aberrant growth signals and loss of growth-inhibiting signals, thus disrupting the cell cycle. Growth signals can be mediated by oversecretion of growth factors, constitutive action of growth factor receptors, or their oncogene surrogates, aberrant expression of such receptors, or the unregulated action of transducer systems downstream from the receptors. Most of these cellular events act in a dominant manner. One or more genes involved in suppression of cell growth may have to be inactivated for malignant transformation or tumor progression to take place. The effects of these genes are usually recessive but there are variations on this theme. Later, with tumor progression the transformed cells may subvert the action of growth-inhibiting cytokines and use them for growth. It is likely that early competence events lead to thyroid cell clonal growth. These cells then become the targets for the random accumulation of supplementary events, including inactivation of tumor suppressor genes which would lead to malignant transformation. It can be inferred that such malignant cells are programmed to alter expression of surface molecules including adhesion proteins as well as to induce proliferation of supporting connective tissue and capillaries which would dictate the biological behavior of these tumors. Figure 9 synthesizes both the defined and putative cellular genetic events involved in thyroid carcinogenesis.

To see a world in a grain of sand
and a heaven in a wild flower
hold infinity in the palm of your hand
and eternity in an hour.

William Blake, *Auguries of Innocence*

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